BARD Final Scientific Report
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BARD Project Number: US-2614-95C

Evaluating Panel: Post Harvest and Food Technologies

Project Title: Bacteriocin Markers for Propionibacteria Gene Transfer Systems

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Continuation of (Related to) Previous BARD Project:
☐Yes ☐No Number:**US-2080-91**

Keywords *not* appearing in the title and in order of importance. Avoid abbreviations. **Antibodies, genetics, preservatives**.

Abbreviations used in the report, in alphabetical order**: AU/ml**, activity units per milliliter; **bp,** base pair; **CFU**, colony-forming units; **CU**, Clemson University; **ELISA**, enzyme-linked immunosorbent assay; **HPLC**, high pressure liquid chromatography; **ISU**, Iowa State University; ***jnG***, jenseniin G gene; **kDa**, kilodalton; **NLA**, sodium lactate agar; **NLB**, sodium lactate broth; **PCR**, polymerase chain reaction; **pI,** isoelectric point; **PLG-1**, propionicin PLG-1; ***plg-1***, propionicin PLG-1 gene; **SDS-PAGE**, sodium dodecyl sulfate polyacrylamide gel electrophoresis; **VI**, Volcani Institute.

Budget: IS: $194,130 US: $103,200 Total: $297,330

Signature: Susan F. Barefoot, Ph.D.
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Research Authority, Principal Institution

B. Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted)	0	4	0	0
Submitted, in review, in preparation	0	3	3	6
Invited review papers	0	0	0	0
Book chapters	0	0	0	0
Books	0	0	0	0
Masters theses	0	3	0	3
Ph.D. theses	0	2	0	2
Abstracts	0	7	2	9
Not refereed (proceedings, reports, etc.)	0	0	0	0

C. Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted	0	0	0	0
Issued (allowed)	0	2	0	2
Licensed	0	0	0	0

D. Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Visits/Meetings	0	1	1	2
Sabbaticals	0	0	0	0
Postdoctorates	0	0	0*	0

***Note:** Dr. Stefanie Baker's postdoctoral fellowship in Dr. Barefoot's laboratory (1998-1999) was supported through this project.

- **Cooperation**, briefly explain whether synergistic, complementary or supportive.

  The investigators were supportive of each other in supplying cultures, information and technical assistance.

E. Abstract

The antibotulinal bacteriocins, propionicin PLG-1 and jenseniin G, were the first to be identified, purified and characterized for the dairy propionibacteria and are produced by *Propionibacterium thoenii* P127 and *P. thoenii/jensenii* P126, respectively. Objectives of this project were to (a) produce polyclonal antibodies for detection, comparison and monitoring of propionicin PLG-1; (b) identify, clone and characterize the propionicin PLG-1 (*plg-1)* and jenseniin G *(jnG)* genes; and (3) develop gene transfer systems for dairy propionibacteria using them as models.

Polyclonal antibodies for detection, comparison and monitoring of propionicin PLG-1 were produced in rabbits. Anti-PLG-1 antiserum had high titers (256,000 to 512,000), neutralized PLG-1 activity, and detected purified PLG-1 at 0.10 μg/ml (indirect ELISA) and 0.033 μg/ml (competitive indirect ELISA). Thirty-nine of 158 strains (most *P. thoenii* or *P. jensenii*) yielded cross-reacting material; four strains of *P. thoenii,* including two previously unidentified bacteriocin producers, showed biological activity. Eight propionicin-negative P127 mutants produced neither ELISA response nor biological activity. Western blot analysis of supernates detected a PLG-1 band at 9.1 kDa and two additional protein bands with apparent molecular weights of 16.2 and 27.5 kDa.

PLG-1 polyclonal antibodies were used for detection of jenseniin G. PLG-1 antibodies neutralized jenseniin G activity and detected a jenseniin G-sized, 3.5 kDa peptide. Preliminary immunoprecipitation of crude preparations with PLG-1 antibodies yielded three proteins including an active 3-4 kDa band. Propionicin PLG-1 antibodies were used to screen a *P. jensenii/thoenii* P126 genomic expression library. Complete sequencing of a cloned insert identified by PLG-1 antibodies revealed a putative response regulator, transport protein, transmembrane protein and an open reading frame (ORF) potentially encoding jenseniin G. PCR cloning of the putative *plg-1* gene yielded a 1,100 bp fragment with a 355 bp ORF encoding 118 amino acids; the deduced N-terminus was similar to the known PLG-1 N-terminus. The 118 amino acid sequence deduced from the putative *plg-1* gene was larger than PLG-1 possibly due to post-translational processing. The product of the putative *plg-1* gene had a calculated molecular weight of 12.8 kDa, a pI of 11.7, 14 negatively charged residues (Asp+Glu) and 24 positively charged residues (Arg+Lys). The putative *plg-1* gene was expressed as an inducible fusion protein with a six-histidine residue tag. Metal affinity chromatography of the fused protein yielded a homogeneous product. The fused purified protein sequence matched the deduced putative *plg-1* gene sequence. The data preliminarily suggest that both the *plg-1* and *jnG* genes have been identified and cloned.

Demonstrating that antibodies can be produced for propionicin PLG-1 and that those antibodies can be used to detect, monitor and compare activity throughout growth and purification was an important step towards monitoring PLG-1 concentrations in food systems. The unexpected but fortunate cross-reactivity of PLG-1 antibodies with jenseniin G led to selective recovery of jenseniin G by immunoprecipitation. Further refinement of this separation technique could lead to powerful affinity methods for rapid, specific separation of the two bacteriocins and thus facilitate their availability for industrial or pharmaceutical uses. Preliminary identification of genes encoding the two dairy propionibacteria bacteriocins must be confirmed; further analysis will provide means for understanding how they work, for increasing their production and for manipulating the peptides to increase their target species. Further development of these systems would contribute to basic knowledge about dairy propionibacteria and has potential for improving other industrially significant characteristics.

F. Achievements

1. Scientific Results and Implications:

a. Polyclonal antibodies for detection, comparison and monitoring of propionicin PLG-1 were produced. *Propionibacterium thoenii* P127 was grown in fed-batch fermentations. Propionicin PLG-1 was isolated by ammonium sulfate precipitation from culture supernates and purified by semi-preparative ion exchange chromatography and reverse-phase HPLC. Polyclonal antibodies reactive to PLG-1 were produced in rabbits by immunizing with the bacteriocin and Freund's complete adjuvant (initial immunization) or Freund's incomplete adjuvant. Anti-PLG-1 antiserum titers were high (256,000 to 512,000) after the third immunization. Anti-PLG-1 antiserum neutralized PLG-1 activity. Detection limits for purified PLG-1 were 0.10 μg/ml (indirect ELISA) and 0.033 μg/ml (competitive indirect ELISA). Culture supernates from 158 strains of classical dairy propionibacteria were tested for cross-reacting protein by the ELISA and for bacteriocin activity by the well diffusion assay. Thirty-nine strains (most *P. thoenii* or *P. jensenii*) yielded cross-reacting material. Only four strains of *P. thoenii,* including two previously unidentified bacteriocin producers, showed biological activity. Eight propionicin-negative P127 mutants and various other organisms produced neither ELISA response nor biological activity. Western blot analysis of supernates detected a PLG-1 band at 9.1 kDa and two additional protein bands with apparent molecular weights of 16.2 and 27.5 kDa. Different bands were seen in different strains. Further analysis of the nature of the cross-reactive proteins is needed.

b. Propionicin PLG-1 polyclonal antibodies cross-reacted with and could be used for detection of jenseniin G. Jenseniin G activity was produced in *P. jensenii/thoenii* P126 fed-batch cultures, was increased at least 5-fold over producer cultures maintained at pH 6.4, and was used to prepare crude (ammonium sulfate-precipitated, dialyzed) jenseniin G. PLG-1 antibodies neutralized jenseniin G activity and detected a jenseniin G-sized, 3.5 kDa peptide. Preliminary immunoprecipitation of crude preparations with PLG-1 antibodies reduced contaminating proteins and yielded three proteins including an active 3-4 kDa band. Further refinement of this technique could lead to a powerful affinity method for rapid, specific separation of jenseniin G, a task that has heretofore proven difficult, and could similarly facilitate PLG-1 purification.

c. **The data preliminarily suggest that the *plg-1* and *jnG* genes have been identified and cloned.** Propionicin PLG-1 antibodies were used to screen a *P. jensenii/thoenii* P126 genomic expression library. Several possible clones were identified with PLG-1 antibodies; complete sequencing of the insert of one clone revealed a putative response regulator, transport protein, transmembrane protein and an open reading frame that potentially encodes jenseniin G. The molecular weight and pI of the deduced amino acid sequence agreed with values reported for jenseniin G. Whether the

clones produce jenseniin G activity is being investigated and expression studies are underway. Further confirmation is required; however, the structural gene and related genes affecting jenseniin G production and transport appear to have been located. Cloning of the putative *plg-1* gene via PCR technology yielded four sequenceable fragments. The 1,100 bp fragment included an open reading frame (ORF) of 355 bp that encoded 118 amino acids; the deduced N-terminus was similar to the known PLG-1 N-terminus and was located at the ORF beginning. The 118 amino acid sequence deduced from the putative *plg-1* gene was larger than PLG-1 possibly due to post-translational modification, maturation and translocation. Biochemical properties deduced for the putative *plg-1* gene product were calculated molecular weight of 12,846 daltons, isoelectric point of 11.7, 14 negatively charged residues (Asp+Glu) and 24 positively charged residues (Arg+Lys). The putative *plg-1* gene was expressed as an inducible fusion protein with a six-histidine residue tag. Purification of the fused protein by metal affinity chromatography yielded a homogeneous product. The sequence of the fused purified protein matched that deduced from the putative *plg-1* gene. Although activity has not been demonstrated for the fused protein product, preliminary indications are that the gene encoding PLG-1 has been cloned.

d. Characterization of the *plg-1* and *jnG* bacteriocin genes and their development as models for dairy propionibacteria gene transfer systems is incomplete. Characteristics identified for the *plg-1* and *jnG* gene products have been described (item c). Although regulatory and processing-related genes for the bacteriocins appear to have been located, these findings must be confirmed. Characterization of the identified genes and confirmation of their identities is in progress and must precede their application in gene transfer models for dairy propionibacteria.

e. Additional results: Mode of action. Most bacteriocins exert activity by forming a pore in the target cell's membrane. No efflux of potassium ions or small molecules such as nucleotides occurred when PLG-1 was applied to target cells, therefore, PLG-1 is not a pore former. PLG-1 halted medium acidification immediately after its addition. Our conclusion is that PLG-1 is not a pore former but inhibits other essential component in the bacterial cell. Simple method of producing PLG-1. A simpler way to grow propionibacteria for production of PLG-1 was investigated. Propionibacteria were grown in sodium lactate media with high concentration of lactate and 0.6% agar in bottles without aeration for 10 days. This method of fermentation is shorter than other methods and gives better yield without the need of expensive equipment. Identification of other propionibacteria bacteriocins. Jenseniin P is an anti-acne bacteriocin produced by *P. jensenii* B1264. Bacteriocin-like activities were identified for *P. thoenii* P15 and P20.

2. Significance:

In earlier research efforts supported by BARD (US-2080-91), propionicin PLG-1 and jenseniin G were the first bacteriocins to be identified, purified and characterized for the industrially important dairy propionibacteria. In this study, demonstrating that antibodies can be produced for propionicin PLG-1 and that those antibodies can be used to detect, monitor and compare activity throughout growth and purification was an important step towards the use of monitoring their concentrations in food systems. The unexpected but fortunate cross-reactivity of propionicin PLG-1 antibodies with jenseniin G led to their application in selectively recovering jenseniin G by immunoprecipitation. Further refinement of this separation technique could lead to powerful affinity methods for rapid, specific separation of the two bacteriocins and thus facilitate their availability for industrial or pharmaceutical uses. Preliminary identification of genes encoding the two dairy propionibacteria bacteriocins has further defined their characteristics. Once their identity has been confirmed, further analysis will provide means for understanding how they work, for increasing their production and for manipulating the peptides to increase their target species. Thus, results presented in this final report amplify the possibilities for using these bacteriocins in foods and in other applications.

3. Potential for Application:

The production of these bacteriocins by food-grade organisms, their stability to heat, to extremes of pH, and to storage conditions, and their spectrum of activity make them good candidates for use as food preservatives. Sufficient data (spectrum, storage stability, production conditions, crude preparations, gross mode of action, purification methods, etc.) have been developed for both bacteriocins. Dr. Glatz previously described the use of milk culture fermentates containing propionicin PLG-1 as a preservative in a dairy product. However, before either purified bacteriocin can be applied to foods, clinical and/or toxicological studies would be required.

4. Details of Cooperation:

The investigators have shared bacterial cultures, techniques and results. Dr. Glatz provided antibodies to Drs. Barefoot and Gollop. The investigators and their co-workers have communicated by telephone, E-mail and surface mail at regular intervals; E-mail has been particularly useful in this regard. Dr. Glatz visited Clemson University to consult with Dr. Barefoot's lab group in 1996. Extensive E-mail consultation occurred between Mr. Jay Leversee (Dr. Glatz's student) and Dr. Stefanie Baker (post-doctoral fellow in Dr. Barefoot's lab) in 1998-1999. Drs. Barefoot. Glatz and Gollop met at the 2nd International Conference on Propionibacteria (Ireland, June 1998) to share results and research procedures and to plan activities for the final year of the project. Dr. Gollop visited Dr. Barefoot's lab (November 1998) to present a seminar, meet with her laboratory staff and share research methods and results.

II. Body of the Final Report

A. Background, Scientific and Agricultural Relevance of the Project:

The industrially significant dairy propionibacteria produce the "eyes" and nutty flavor in Swiss cheeses, serve as silage adjuncts and probiotics, participate in rum and other natural fermentations, make vitamin B_{12}, produce propionic acid and other natural preservatives including bacteriocins. Three bacteriocins produced by dairy propionibacteria were identified by the investigators (Lyon and Glatz, 1991, 1993; Grinstead and Barefoot, 1992; Ratnam et al., 1999). Two, propionicin PLG-1 produced by *P. thoenii* P127 (ATCC 4874) and jenseniin G produced by *P. jensenii/thoenii* P126 (ATCC 4827), were isolated from culture supernates and their characteristics were determined. Both propionicin PLG-1and jenseniin G showed promise as antibotulinal food preservatives (Garren et al, 1995).

Genetic manipulation has been only marginally successful and means of introducing foreign DNA into the propionibacteria are needed. In this project we proposed to use genes for the bacteriocins, propionicin PLG-1 (*plg-1*) and jenseniin G (*jng*) produced by dairy propionibacteria, as models and food-grade markers in developing gene transfer systems. Objectives were to (a) produce polyclonal antibodies for detection, comparison, and monitoring of propionicin PLG-1; (b) identify, clone and characterize the *plg-1* and *jnG* genes; and (c) develop gene transfer systems for dairy propionibacteria using those genes as models.

Previous annual reports (Barefoot et al., 1997, 1998) detailed the progress of this project and, except for the following summary, will not be repeated. Glatz and co-workers refined methods for large-scale production of propionicin PLG-1 in NLB fed-batch producer cultures (1.2% initial lactate, 0.6% lactate fed every 12 hours; Paik and Glatz, 1997), for purifying large quantities to homogeneity [$(NH_4)_2SO_4$ precipitation, diaysis, ion exchange chromatography, preparative C_{18}HPLC] (Paik and Glatz, 1995) and for preparing antibodies. Barefoot and co-workers (Ratnam et al. 1999) identified, preliminarily purified and characterized a new bacteriocin jenseniin P with anti-acne activity. Jenseniin P, produced by *P. jensenii* B1264, inhibited propionibacteria and lactic acid bacteria, had a bactericidal mode of action against *L. delbrueckii* subsp. *lactis* ATCC 4797, a pI between 3 and 3.5 and a molecular weight between 6 - 9 kD.

Barefoot and colleagues unsuccessfully attempted to sequence jenseniin G (Garver, 1999) and defined additional characteristics (partial composition; size, 3,648 kDa), conditions for improved production (NLB culture, late stationary

growth, pH 6.4; Ekinci and Barefoot, 1999) and purification methods (reverse phase-HPLC; Garver, 1999). Difficulties in sequencing and inability to develop a homologous probe necessitated alternatives for determining the location of the *jnG* gene. Gollop and his co-workers introduced kanamycin resistance from pEK0 into *P. thoenii* P127 by biolistic transformation but could detect no plasmid in two resistant mutants. They constructed a possible shuttle vector using pBR322 and native *Propionibacterium* plasmid but were not successful in transforming propionibacteria. Gollop and Lindner (unpub. data) examined effects of nisin and propionicin PLG-1 on efflux of P_i, K^+, UV-absorbing materials, intracellular ATP and acid production by sensitive cells and determined that their modes of action differ.

During the final project year, the investigators focused on producing and characterizing polyclonal antibodies for detection, comparison, and monitoring of propionicin PLG-1 (ISU) and on identifying and characterizing the *plg-1* (VI) and *jnG* (CU) genes.

B. Methodologies and Materials:

Microorganisms and media (ISU). Propionibacteria strains were propagated in sodium lactate broth (NLB; Hettinga et al, 1968) at 32C as described previously (Lyon and Glatz, 1991). Indicator strain *Lactobacillus delbrueckii* subspecies *lactis* ATCC 4797 and other lactic acid bacteria tested were propagated as described previously (Hsieh et al., 1996). Other bacterial strains were propagated in Tryptic Soy Broth (TSB) (Difco Laboratories, Detroit, MI) statically at 37°C. Long-term storage of cultures was at -80C in the appropriate medium containing 20% glycerol.

Cultures used to test for antibody cross-reactivity were grown statically at the appropriate temperature. Supernatant samples taken after 5 or 12 days of incubation were filtered sterilized and stored at –20C until required. Species and numbers of strains of cultures tested for cross-reactivity include: *Propionibacterium thoenii* (8), *P. jensenii* (26), *P. acidipropionici* (21), *P. freudenreichii* subsp. *shermanii* (79), *P. freudenreichii* subsp. *freudenreichii* (14), uncharacterized *Propionibacterium* spp. (10), bacteriocin-negative mutants of *P. thoenii* P127 (8), *Lactobacillus bulgaricus* (3*), Lb. delbrueckii* (1), *Lb. casei* (1), *Lactococcus cremoris* (1), *Lc. diacetylactis* (1), *Lc. lactis* (1*), Streptococcus thermophilus* (1), *Bacillus cereus* (1), *Escherichia coli* (1), *Pseudomonas aeruginosa* (1), and *Staphylococcus aureus* (1).

Propionicin PLG-1 production and purification (ISU). *P. thoenii* P127 was grown in 14-day fed-batch fermentations, cells were removed by centrifugation (8,000 x *g*, 30 min) and propionicin PLG-1 was harvested from the cell-free supernates and purified using ammonium sulfate precipitation, ion exchange chromatography, and reverse-phase HPLC as described previously

(Paik and Glatz, 1995). Purification was confirmed by SDS-PAGE of the sample followed by silver staining and overlaying the gel with the indicator organism to confirm biological activity of stained bands (Bhunia and Johnson, 1992). Purified PLG-1 samples were combined and stored at –80°C.

Production and Preparation of Jenseniin G (CU). Batch and fed-batch fermentations were conducted without aeration at 32C under flowing CO_2 (0.4 L/hr) in 1 L screw top bottles. A 2 % (v/v) inoculum of a 44-hr broth culture of *P. jensenii (thoenii)* P126 was used to inoculate each liter of sterile NLB. The jenseniin G producer was propagated in fed-batch fermentations (14 days, NLB) containing 1.2% sodium lactate under flowing CO_2. After 48 hr incubation, sodium lactate was added every 24 hr to a final concentration of 0.3%. 20-fold concentrated NLB (without lactate) was added at day 7 to replenish approximately 15% of the other nutrients (Paik and Glatz, 1997). Aliquots (25 ml) of producer cultures were removed daily for bacteriocin assays, turbidity ($A_{600, 1cm}$), viable plate counts (CFU/ml), and organic acid quantitations via HPLC (Garcia and McGregor, 1994).

Crude jenseniin G was prepared by freeing producer cultures of cells by centrifugation (15 min, 4C, 10,000 x *g*) and passage through 0.45-μm filters. Filtrates were concentrated 50-fold by precipitation with 80% $(NH_4)_2SO_4$ (24 hr, 4C). Precipitated proteins were collected by centrifugation at 10,000 x *g* (60 min, 4C). Pelleted proteins were resuspended in sterile distilled water at 1/50 original volume.

Bacteriocin Assays (ISU, CU, VI). Propionicin PLG-1 activity, expressed in activity units per ml (AU/ml), was determined using serial dilutions of samples tested in duplicate in the agar well diffusion assay (Hsieh et al., 1996). Jenseniin G activity was determined by the critical dilution method (Grinstead and Barefoot, 1992). *Lb. delbrueckii* subspecies *lactis* ATCC 4797 was the indicator organism. Bacteriocin titers were calculated from the reciprocal of the highest dilution producing a detectable zone of inhibition and were reported in AU/ml of the original sample. To test for inhibition of bacteriocin activity by antibodies, serial dilutions of PLG-1 were mixed with equal volumes of antiserum in the well. Activity was compared to that in control wells containing distilled water or preimmune serum mixed with bacteriocin. Activity of crude jenseniin G (100 AU/ml or 1000 AU/ml) in the presence or absence of anti-propionicin PLG-1 antibodies was assayed by a spot-on-lawn method (Weinbrenner et al, 1997).

Immunization and Serum Harvest (ISU). All procedures were conducted at the Iowa State University Cell and Hybridoma Facility. Three New Zealand white rabbits were immunized both subcutaneously and intramuscularly with 1 ml of antigen solution containing ~200 μg propionicin PLG-1 and either Freund's

complete adjuvant (initial immunization) or Freund's incomplete adjuvant (booster immunizations at 4-week intervals). After titers reached acceptable levels, one rabbit was chosen for antibody production and maintained for production bleeds (30-day cycle of an immunization followed by three 50-ml bleeds at 10-day intervals). Blood samples were refrigerated for 24 h, centrifuged (450 x *g*; 10 min) at room temperature, and the serum was stored at -20C.

Indirect ELISA (ISU). For antisera titration, the protocol for the indirect ELISA was similar to Fan and Chu (1984). Goat anti-rabbit IgG-HRP conjugate (1:5,000 dilution; Sigma, St. Louis, MO) was the secondary antibody, and the reporter substrate was *o*-phenylenediamine (OPD, Sigma). Absorbance (492 nm) of each well was read with a Bio Kinetics EL 340 microtiter plate reader (Bio Tek Instruments, Inc., Winooski, VT). Titer of the antiserum was defined as the reciprocal of the highest dilution of immune serum that produced an absorbance in the ELISA at least twice as high as the average absorbance for all dilutions of pre-immune serum.

SDS-PAGE (ISU). Gel electrophoresis was performed using the buffer system described by Laemmli (1970) in 10-20% gradient polyacrylamide gels (Mini-PROTEAN II Ready Gels; Bio-Rad Laoratories, Hercules, CA). Proteins were separated at 180 V for 45 min using the Bio-Rad mini-PROTEAN II electrophoresis apparatus. Gels were fixed in 30% ethanol-10% glacial acetic acid and silver stained per the manufacturer's instructions (Bio-Rad).

Western Blots (ISU, CU). The Western blot method of Burnette (1981) was used to investigate individual proteins able to bind anti-PLG-1 antibodies in the antiserum. Proteins were resolved by SDS-PAGE or on precast Tris-glycine 10-20% polyacrylamide gels (Bio-Rad Laboratories) and were transferred to a polyvinyl difluoride (PVDF) membrane, Immobilon-P (Millipore), using a Bio-Rad MiniTransblot apparatus. Incubations with primary antibody (rabbit anti-serum) and secondary antibody (goat anti-rabbit IgG horseradish peroxidase conjugate or alkaline phosphatase conjugated goat anti-rabbit immunoglobulin G from Bio-Rad) were at room temperature for 1 hr with gentle agitation. Secondary antibody alone and preimmune serum (PIS) as the primary antibody were included as negative controls. Desired proteins were detected using SigmaFAST™ 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) chromogenic substrate or with diaminobenzidine (Sigma Fast DAB).

Immunoblotting Proteins (Colony Blot) (ISU). The colony blot assay was modified from Noel et al. (1996). Organisms were grown on sodium lactate agar (NLA) for 120 hr at 32C. A moist nitrocellulose membrane disk was pressed

gently onto the colonies and incubated (45 min, room temperature), removed and reacted with primary and secondary antibodies. Antibodies bound to colonies were visualized with DAB.

Library Construction and Screening (CU). Genomic DNA isolated from *P. thoenii/jensenii* P126 was partially digested with *Sau*3AI and resolved on a 0.6% agarose gel. Fragments ranging in size from 6.6 to 9.4 kb were purified and packaged inside λ phage particles using the ZAP Express predigested vector/ Gigapack III Gold cloning kit (Stratagene). Plaque lifts were performed as described in the *pico*Blue™ immunoscreening kit instruction manual (Stratagene). Lifts were screened with anti-propionicin PLG-1 3144 primary antibody supplied by Dr. B.A. Glatz. Following identification of positive plaques, phagemids containing desired insert(s) were excised from the phage genome according to the ZAP Express predigested vector/Gigapack III Gold cloning kit instruction manual (Stratagene).

DNA Sequencing and Analysis (CU). *Sal*I subclones of an excised phagemid, p5B, were sequenced at the Davis Sequencing Automated DNA Sequencing Facility (Davis, CA). Custom oligonucleotide primers were obtained from Integrated DNA Technologies (Coralville, IA). DNA sequences were analyzed using a demonstration version of Sequencher 3.1.1 for MacIntosh.

Immunoprecipitation of Jenseniin G (CU). Anti-propionicin PLG-1 antibodies were attached to Dynabeads® Protein A (Dynal, Inc., Lake Success, NY) per the manufacturer's instructions. Jenseniin G was added to antibody-conjugated beads and incubated with slow tilt rotation (1 hr, room temperature). The beads were washed three times with sodium-phosphate buffer (pH 8.1). Washed beads were resuspended in the same buffer, an equal amount of 2-fold sample buffer (0.063 M Tris, 25% glycerol, 2% SDS, 0.0125% bromophenol blue, 0.36 M β-mercaptoethanol, pH 6.8) was added, and samples were boiled and loaded in duplicate on a precast 10-20% polyacrylamide Tris-tricine gel (Bio-Rad). Half the gel was stained with Coomassie blue (0.025% Coomassie blue G-250, 10% acetic acid); the other half was overlaid with MRS soft agar (0.75% agar) seeded with the indicator organism.

Isolation of *P. thoeni* P127 Genomic DNA (VI): Genomic DNA from over-night NLB *P. thoenii* P127 cultures (32C) was isolated by the method of Flamm et al (1984).

Synthetic Primers (VI). The sequence for the 10 N-terminal amino acids of propionicin PLG-1 (Paik and Glatz, 1995) and the codon usage calculated from known *Propionibacterium* genes in the Genebank database were used to construct 2 oligo-nucleotide (oligos) primers. Oligos were designated as "oligo-sense" by the sequence of amino acids 1-7 (5` AACGTSGAYRCCCGSACCGC 3`) and

"oligo-antisense" according to the sequence of amino acids 5-1 (5` SCGGGYRTCSACGTT 3`). To isolate the gene encoding propionicin PLG-1 (*plg-1*), the "oligo-sense" primer and a 12-nucleotide random primer purchased from New England Biologicals (MA, USA) were used for PCR reactions at an annealing temperature of 55°C for 35 cycles. The "oligo-antisense" and the 12-nucleotide random primers were used to isolate the sequence upstream of the *plg-1* gene.

Cloning and Transformation (VI). PCR-amplified products were separated on 1% agarose gels. Fragments were extracted from gels using the QIAquick gel extraction kit (Qiagen, Germany) and ligated to the pGEMT cloning vector (Promega, USA). Ligation mixtures were introduced into *E. coli* JM109 cells by electroporation. Plasmid from positive colonies (blue white selection) was prepared using the Miniprep system (Qiagen) and sequenced. Sequencing was performed in the DNA Analysis Unit at the Life Sciences Institute (The Hebrew University, Jerusalem, Israel).

Expression of Propionicin PLG-1 in *E. coli* (VI). PCR was used to incorporate a *Nde*I site at the translational start codon (ATG) upstream of PLG-1, and a *Xho*I site downstream of the last nucleotide before the PLG-1 stop codon. The template for PCR amplification was the cloned *plg-1* plasmid; the primers were "PLG-Exp-S" – 5' GGAATTCCATATGAACGTCGACG 3' and "PLG-Exp-E" - 5' CCGCTCGAGGTCCGGAGAATTCGT 3' (underlined letters indicate the *Nde*I and *Xho*I restriction sites, respectively). Amplification was for 35 cycles at an annealing temperature of 55C. The PCR product and pET22b expression vector (Novagene, USA) were digested with *Nde*I and *Xho*I and ligated. The ligation mixture was introduced into *E. coli* DH5α by electroporation. Plasmid from colonies growing on media containing ampicillin (100 μg/ml) was isolated by the Miniprep system (Qiagen) and confirmed to contain the *plg-1* gene by PCR. Recombinant clones were introduced into the expression host strain *E. coli* ER2566 containing the chromosomal T7 RNA polymerase gene. Exponential growth phase (OD_{600nm}=0.6-1.0) cultures were induced with 1mM IPTG (3 hours). Cell extracts were analyzed for expression by SDS-PAGE.

Purification of Recombinant Proteins (VI). Recombinant proteins expressed in 100 ml of induced culture were purified with a metal affinity chromatography system by the manufacturer's instructions (Qiagen). Recombinant protein was purified in either native conditions or denaturing conditions. Purity was analyzed by SDS-PAGE. Purified proteins were injected into two rabbits for production of antibodies against PLG-1.

C & D. Results & Discussion:

Increasing Jenseniin G Production. Batch and fed-batch cultures of the jenseniin G producer, *P. jensenii/thoenii* P126, were monitored for viable counts,

turbidity, organic acids and bacteriocin production. Viable counts in static and fed-batch cultures reached $1.2x10^9$ CFU/ml and $5.4x10^9$ CFU/ml, respectively, during late exponential/early stationary phase (3 days) (Fig. 1). Populations of viable cells in fed-batch fermentations remained constant throughout incubation; after day 6 those in static fermentations dropped to $1.5x10^7$ CFU/ml. Though populations did not change considerably, maximum turbidity of fed-batch cultures was 4-5 fold higher than batch cultures (Fig.1b). Increased turbidity in fed-batch cultures was not because of added lactate since adding sodium lactate to the culture medium did not increase turbidity. Differences may have been partially due to changes in culture absorptivity. Changes in cell shape and size during growth are characteristic of propionibacteria. Cells of *P. jensenii* B1264 in NLB cultures change from primarily rod-shaped to coccoid as they enter stationary phase growth (Graves, 1998). Production of polysaccharides may also have contributed to increased turbidity. Many strains of *P. thoenii* and *P. jensenii* produce extracellular slime or polysaccharide that makes cultures in liquid medium quite viscous (Skogen *et. al*., 1974); visual examinations indicated that *P. jensenii/ thoenii* P126 produced a slime-like material. EPS yield generally is not a direct function of growth but is affected by fermentation conditions (temperature, time) and medium composition (Jutta, 1995).

Batch and fed-batch cultures completely utilized lactate within 48 hours (Fig. 2). Except for day 11, lactate supplied at each feeding was consumed in fed-batch fermentations (Fig. 2b). Concentrations of organic acids increased throughout fed-batch fermentations. In fed-batch cultures, the maximum levels of organic acids were 35.1 mg/ml of propionic acid and 9.4 mg/ml of acetic acid; propionic and acetic concentrations peaked at about 4.9 and 1.5 mg/ml in batch cultures, respectively. Organic acids did not contribute to bacteriocin activity since they were removed during ammonium sulfate precipitation. More jenseniin G was produced in fed-batch cultures than in batch cultures (Fig. 1**)**. Highest bacteriocin activity was detected at day 12 (384 AU/ml) in fed-batch fermentations and at day 9 (21.33 AU/ml) in batch cultures. Activity declined within a day after maximum titers were detected but was not due to adsorption to producer cells (Ekinci and Barefoot, 1999). Decreases may be due to protein aggregation or proteolytic degradation. Similar bacteriocin instability in cultures has been noted for lactacin B (Barefoot and Klaenhammer, 1984), amylovorin L471 (De Vuyst *et. al*., 1996) and propionicin PLG-1 (Paik and Glatz, 1997) and has been attributed to aggregation, proteolytic degradation or adsorption to producer cells.

Maximum activity in concentrated supernates from fed-batch cultures (384 AU/ml) at day 12 was 18-fold higher than activity from static cultures or

fermenter cultures without pH control, and 2.4-fold higher than fermenter cultures held at pH 6.4 (Fig. 3). Production of jenseniin G can be increased by cultivating *P. jensenii/thoenii* P126 in fed-batch batch fermentations.

Ekinci and Barefoot (1999) previously demonstrated that jenseniin G production is pH-dependent. Maximum activity (20 AU/ml) was detected in *P. thoenii (jensenii*) P126 batch and fermenter cultures without pH control. Production of jenseniin G was examined at controlled pH (pH 5.75, 6.00, 6.40 or 7.00) in fermenter cultures. Highest activity (160 AU ml^{-1}) was detected during stationary phase producer cultures maintained at pH 6.40, suggesting that reactions necessary for jenseniin G synthesis and/or release are dependent upon one or more pH sensitive components. Given its pH-dependence, even higher bacteriocin production might be obtained in fed-batch cultures maintained at pH 6.4. Fed-batch fermentation shows promise as a method for obtaining high concentrations of industrially significant bacteriocins from dairy propionibacteria

Production and Purification of Propionicin PLG-1 (ISU). Several large-scale fed-batch fermentations of *P. thoenii* P127 were conducted to produce 2,600 mg of partially purified, ammonium sulfate precipitated propionicin PLG-1. Propionicin activity eluted from a semi-preparative scale carboxymethyl-Sepharose column between 0.50 and 0.75 M in a sodium chloride gradient. This material was concentrated and applied to a C_{18} reverse-phase HPLC semi-preparative column (YMC Inc., Wilmington, NC). Activity eluted between 25 and 30% in a 2-propanol gradient in 0.1 % acetic acid. A single peak was observed at 25% 2-propanol. This material was termed purified propionicin PLG-1 and was used for antibody production. Activity yield was 7.4% and increase in purification over partially purified bacteriocin was about 5-fold.

Antibody Production (ISU). Polyclonal antibodies against propionicin PLG-1 were obtained after the primary immunization and one booster immunization (34 days). ELISA titers of 1:256,000 to 1:512,000 were stable throughout the antiserum production cycle (152 days). Antiserum from one bleed of one rabbit was used for all experiments.

Activity Reduction of Propionicin PLG-1 by Antiserum(ISU). Antiserum completely inhibited purified propionicin PLG-1 (100 AU/ml biological activity) in the well diffusion assay. It reduced biological activity of two partially purified samples with activities of 1,280 and 10,240 AU/ml, by over 98%. The high protein content of the partially purified samples may have interfered with antiserum binding to PLG-1. Preimmune serum had no effect on biological activity. These results suggest that specific antibodies in the antiserum reduced the biological activity of PLG-1.

Propionicin PLG-1 Detection in Fermentation Supernates (ISU). A fed-batch fermentation was run under standard conditions for 408 h. Supernatant samples taken about every 12 hours were assayed for PLG-1 content (indirect ELISA) and bacteriocin activity (well diffusion assay). The culture reached stationary phase at about 60 h. Bacteriocin activity was first detected in supernates at 217 h and was at maximum between 265 and 372 hr (Fig. 2A). In contrast, supernatant samples from as early as 5 h of incubation gave a color response in the ELISA (Fig. 2B). Absorbance peaked at 36 hours and remained high for the duration of the fermentation. Quantitation of propionicin PLG-1 was not possible because the response was beyond the linear detection range of the standard curve. Neither medium components nor propionic or acetic acids gave reactions in the indirect ELISA.

The positive response of early supernates in the ELISA suggested that proteins binding the antibodies were primary metabolites produced during the logarithmic growth phase. Assay values remained steady or decreased somewhat as the culture reached the stationary growth phase and bacteriocin activity titer in supernates increased. Possibly the antibodies interacted with not only the active PLG-1 but also inactive forms of PLG-1, e.g., either prepeptide or multimeric forms of the bacteriocin or associations of propionicin with other proteins.

Immunoblotting of Propionicin PLG-1 and Fermentation Supernates (ISU). Concentrated purified PLG-1, partially purified PLG-1, 72-h and 312-h supernates from the above fermentation, and a supernate from a previous fermentation with high bacteriocin activity were separated by SDS-PAGE and probed with anti-PLG-1 antiserum (Fig. 3). Purified PLG-1 contained a doublet band of apparent molecular weight 9.1 kDa and closely matched the molecular weight of PLG-1 (9,328 Da) reported by Paik and Glatz (1995). A second, diffuse protein band of approximately 16.2 kDa was also visible. Others have noted that the hydrophobic nature of some bacteriocins caused inconsistent protein migration in SDS-PAGE separations (Nettles and Barefoot, 1993). Hydrophobic interaction could account for this diffuse band with larger apparent molecular weight.

Two reactive protein bands of approximately 16.2 and 27.5 kDa were seen in the 72-hr supernate. These bands may be multimers of the PLG-1 protein, or they may be hydrophobic associations of PLG-1 with other proteins in the supernatant that still allow antibody binding to the PLG-1 molecule. At 312 hr, only the 16.2 kDa band was present in the supernatant; no distinct bands representing smaller proteins were seen. This sample did have low, but detectable, bacteriocin activity (20 AU/ml). The supernatant sample from a

separate fermentation with higher bacteriocin activity (40 AU/ml) contained a 16.2 kDa band, a faint 27.5 kDa band, and a very faint 9.2 kDa band that was visible to the eye, but not visible in the scanned image. It is possible that the propionicin PLG-1 molecule (9.1 kDa) can form dimers (about 16 to 18 kDa), trimers (27 kDa), or even larger multimeric forms. The multimeric forms may appear first during growth, and release the monomer. Upon storage or concentration, the monomer may reform multimers. The monomer is biologically active. Biological activity of multimeric forms is yet to be determined precisely, but seems low. The partially purified PLG-1 sample contained proteins concentrated from the 336-hr supernate by ammonium sulfate precipitation. It contained intense bands of 9.1 and 16.2 kDa and four other distinct bands with approximate weights of 10.7, 12.8, 40.5, and 72.1 kDa. The 27.5 kDa band, not present in the 312-h supernatant, was also missing here. Ammonium sulfate precipitation possibly caused PLG-1 to form hydrophobic associations with other small proteins (10.7 and 12.8 kDa bands). The larger molecular weight bands (40.5 and 72.1 kDa) may represent associations with larger proteins or further polymerization of PLG-1 beyond the trimer form. Possible aggregation of bacteriocin protein with other substances to form large macromolecules has been suggested previously for PLG-1 (Lyon and Glatz, 1993).

Several bacteriocins of gram-positive bacteria are ribosomally synthesized as biologically inactive precursor peptides (prepeptides) that are later modified enzymatically into the active bacteriocin form (propeptide) by removal of a peptide leader sequence (Jack et al., 1995). This might offer another explanation for the larger proteins bound by the anti-PLG-1 antibodies that were present in the fermentation supernatant samples. Bhunia (1994) reported similar cross-reaction of anti-pediocin monoclonal antibodies with large proteins in fermentation supernates of *Pediococcus acidilactici* and suggested that these might be biologically inactive prepediocin proteins that disappeared as the culture aged.

One or both of the larger proteins in the PLG-1 samples may be a prepeptide that contains the PLG-1 amino acid sequence recognized by the antibodies in addition to a nonspecific leader sequence. As the prepeptide is enzymatically cleaved, the PLG-1 molecule appears as a smaller peptide. The cleavage may even be a two-step process that first transforms the prepeptide from the original 27.5 kDa size to 16.2 kDa, followed by another cleavage to the active 9.1 kDa propeptide form. All forms of the bacteriocin would theoretically contain the PLG-1 propeptide that binds the antibodies. The presence of the 16.2 kDa precursor in the purified PLG-1 sample might indicate low levels of

copurification of the precursor along with the active propeptide form. Another possibility that must be considered is specific or nonspecific binding of the antibodies to protein(s) that co-purified with PLG-1.

Cross-reactive Proteins from Other Bacteria (ISU). Supernates from 158 strains of five *Propionibacterium* species were tested for bacteriocin activity in the well diffusion assay and for cross-reaction with anti-propionicin PLG-1 antiserum. After 5 days of incubation, only *P. thoenii/jensenii* P126 was active in the well diffusion assay (titer of 10 AU/ml). This strain produces the bacteriocin jenseniin G (Grinstead and Barefoot, 1992). After 12 days of incubation, four strains of *P. thoenii* (P15, P20, P126, and P127) yielded bacteriocin titers of 20 AU/ml. This observation provided the first report of bacteriocin activity for strains P15 and P20.

Cross-reactions with anti-propionicin antibodies in the ELISA are summarized in Table 1. Supernates with absorbances greater than 10 times the negative controls were considered to be cross-reactive. At least one strain of each *Propionibacterium* species cross-reacted in this test. All strains of *P. thoenii* were cross-reactive at one or both of the sampling times. A majority of strains of *P. jensenii* were cross-reactive; these had very high absorbance values. Most strains of *P. acidipropionici* and *P. freudenreichii* were non-cross-reactive. Bacteriocin-negative mutants of *P. thoenii* P127 did not cross-react; neither did strains of other bacteria.

Western Blot Analysis of Cross-reactive Supernatants. Supernatants that cross-reacted in the ELISA and samples active in the well diffusion assay were tested in the western blot procedure. Selected nonreactive samples, including bacteriocin-negative mutants of strain P127, were included as negative controls (Table 2). Most supernatants contained a 16.2 and/or a 27.5 kDa protein band. These bands may be the 16.2 and 27.5 kDa proteins previously observed in supernates from *P. thoenii* P127. All *P. thoenii* supernates contained the 16.2 kDa protein; strains P30 and P38 also contained the 27.5 kDa protein. In contrast, the 27.5 kDa protein band was seen in all but one *P. jensenii* supernate. Only some *P. jensenii* supernates also contained the 16.2 kDa protein. No supernates, not even those that had biological activity in the well diffusion assay, had a visible 9.1 kDa band, which is hypothesized to represent the active monomer form of PLG-1. The titers of these active samples were low (20 AU/ml). Three of five bacteriocin-negative P127 mutants showed no protein bands, but the other two mutants showed very faint bands at the 27.5 kDa position.

These results suggest that many propionibacteria produce similar 16.2 and 27.5 kDa proteins able to bind antibodies. These large proteins may be responsible for the cross-reactivity seen in the indirect ELISA. They may be

either precursor or multimer forms of PLG-1; these strains may lack mechanisms to process the bacteriocin into its active form. The bacteriocin-negative *P. thoenii* P127 mutants may have lost the ability to produce the original precursor or multimer forms of PLG-1 and therefore do not produce active PLG-1.

Colony Blot Analysis (ISU). Six clones of strain *P. thoenii* P127 produced very strongly positive reactions in the colony blot. No visible reactions were seen for any clones of two strains of *P. freudenreichii* and *P. acidipropionici* and one strain of *Bacillus cereus* used as negative controls. All clones of bacteriocin-negative P127 mutants gave positive reactions; these were strongly positive for all clones of one isolate, but were weak for the clones of a different isolate. These results suggest that the bacteriocin-negative mutants may produce some amount of one or more forms of cross-reactive proteins when grown on solid medium. Perhaps in liquid culture, these proteins are not produced in as large quantities or they may be diluted to undetectable levels. After protein transfer to the nitrocellulose membrane, a thin layer of agar seeded with indicator organism was overlaid onto the base agar layer to determine whether active bacteriocin was produced by clones. No zones of inhibition were seen, even for the P127 clones. It is possible that the 5-day incubation time was not sufficient for *P. thoenii* P127 to produce detectable levels of active PLG-1. The other organisms were not known to produce active bacteriocins.

Anti-propionicin PLG-1 3144 Antibodies Cross React with Jenseniin G (CU). The following results were obtained by Dr. Stefanie Baker, a BARD-supported post-doctoral fellow in Dr. Barefoot's laboratory. To determine if the anti-propionicin PLG-1 antibodies recognized jenseniin G, mixtures of crude jenseniin G and preimmune serum (PIS) or test serum were assayed for bacteriocin activity (Table 3). A dilute preparation of jenseniin G (100 AU/ml) was completely inhibited by all levels of antibody tested but was not inhibited by preimmune serum. When a more concentrated sample of jenseniin G was tested (1000 AU/ml), inhibition decreased as the antibody concentration decreased indicating that inhibition of activity could be overcome by adding excess jenseniin G.

Western blot analysis was performed to determine if the anti-propionicin PLG-1 antibodies are specific for jenseniin G. When a blot containing HPLC-purified jenseniin G was probed with anti-propionicin PLG-1 antibodies, a 3.5 kDa protein was detected; however, several proteins were detected on the blot containing crude jenseniin G. No proteins were detected when the blots were probed with the secondary antibody alone or with the preimmune serum. These results suggest that the anti-propionicin PLG-1 antibodies do recognize jenseniin G, but they may also cross react with other proteins

present in the crude preparation. The same results were observed for purified and crude preparations of propionicin PLG-1.

Immunoprecipitation of Jenseniin G (CU)**.** Jenseniin G was partially purified from a crude preparation by immunoprecipitation using anti-propionicin PLG1 antibodies attached to magnetic Dynabeads® Protein A (Dynal, Inc.). Only three protein bands were observed after Coomassie blue staining of the samples resolved by SDS-PAGE compared to multiple bands observed before immuno-precipitation. Overlaying a duplicate gel with MRS soft agar seeded with the indicator culture yielded a zone of inhibition centered at ~3-4 kDa. This successful immunoprecipitation paves the way for future affinity methods for efficient purification of the difficult-to-purify jenseniin G peptide.

Identification of Putative *jenG* Clones (CU). Screening of the *P. jensenii/ thoenii* P126 genomic library with anti-propionicin PLG-1/anti-jenseniin G antibodies yielded nine possible jenseniin G-harboring clones. Restriction mapping of phagemids excised from seven of the clones revealed that many of the clones were identical; therefore, only three were chosen for further analysis. *E. coli* transformed with phagemid 5B grew more slowly than the others; therefore, *Sal*I subclones of p5B were constructed and the entire 7.0 kb insert was sequenced. A BLAST search identified open reading frames with significant homology to a response regulator, a transmembrane protein, and an ATP binding cassette (ABC) transporter. Although the genetic organization of bacteriocin operons varies in different organisms, genes encoding regulators and transporters are usually located in close proximity to the bacteriocin structural gene (Ennahar et al, 2000). Further analysis revealed a 192 bp open reading frame with a deduced amino acid sequence which shares features with other Gram-positive bacteriocins. First, many bacteriocins contain a double glycine motif where cleavage forms the mature bacteriocin from the prebacteriocin. A double glycine motif is present at residues 24 and 25 of the deduced amino acid sequence. Secondly, many Gram-positive bacteriocins, including jenseniin G, are small, cationic peptides. The molecular weight of the putative mature peptide is 3931 daltons and the isoelectric point is approximately 11. These values are in agreement with the molecular weight and pI previously reported for jenseniin G (Grinstead, 1993; Garver, 1999).

Various methods exist to identify the gene responsible for encoding a protein of interest. One method is to purify the protein, generate antibodies against it, and use the antibodies to screen an expression library. Antibodies raised against propionicin PLG1, a bacteriocin produced by the closely related strain *P. thoenii* P127, were used to screen a *P. jensenii/ thoenii* P126 genomic expression library. Several possible clones were identified and the insert of one was completely sequenced. Sequence analysis revealed that this clone has many similarities with other bacteriocin operons. A putative response regulator,

transport protein and transmembrane protein were identified based on protein homology. An open reading frame that potentially encodes jenseniin G was also identified. Both the molecular weight and the isoelectric point of the deduced amino acid sequence agree with values reported for jenseniin G. Expression of the open reading frames in *E. coli* is currently in progress.

Cloning the Propionicin PLG-1-encoding Gene (VI). PCR reactions were conducted to clone the *plg-1* gene using chromosomal DNA from the PLG-1 producer strain *P. thoenii* P127 as template. Primers were the "oligo-sense" and random primers. Four fragments were observed on agarose gels (Fig 4, lane 2). The fragments differed in size from the PCR primers (Fig 4, Lane 3). The fragments were cloned into the pGEMT cloning vector and sequenced.

The following data point to the conclusion that fragment 1 (1,100 bp) contained the *plg-1* gene: (a) The fragment contains an open reading frame of 355 bp (Fig 5a) that encodes 118 amino acids (Fig 5b). This open reading frame is larger than the 99 amino acids previously reported for propionicin PLG-1 (Paik and Glatz, 1995). (b) The N-terminus of the open reading frame is similar to the known N-terminus of PLG-1. (c) The DNA sequence corresponding to the PLG-1 N-terminus was located at the beginning of the gene.

The translated protein from the open reading frame of the *plg-1* gene contained 118 amino acids and is larger than the empirical size of 99 amino acids reported for PLG-1 (Paik and Glatz, 1995). This size mismatch may derive from modification, maturation and translocation processes whereby 19 amino acids are removed from the polypeptide. Two of the 10 amino acids deduced from the *plg-1* sequence differed from the 10 N-terminal amino acids reported for the PLG-1 protein. They were arginine instead of alanine in position 7 and arginine instead of threonine in position 9. Arginine responds more weakly than other amino acids during protein sequencing. Thus, arginine may be easily mis-identified as another amino acid.

To isolate the region upstream of the *plg-1* gene, the "oligo-antisense" primer and the random primer were used for PCR reactions and the chromosomal DNA of *P. thoenii* P127 was used as template (data not shown). A single fragment (1000 bp) resulting from the PCR reaction was cloned into the pGEMT cloning vector and sequenced (Fig 5). An ATG translational start codon corresponding to a methionine was detected at the beginning of the *plg-1* gene (Fig 5). This methionine is probably removed during the maturation of PLG-1. Theoretical promoter regulatory sites were identified and highlighted in the sequence upstream of *plg-1* (Fig 5). Biochemical properties calculated from the sequence of the *plg-1* gene are as follows: a calculated molecular weight of 12,846 daltons, an isoelectric point of 11.7 pH, 14 negatively charged residues

(Asp+Glu) and 24 positively charged residues (Arg+Lys). These values deviate substantially from those previously reported for PLG-1 (Paik and Glatz, 1995) but can be explained by post-translational processing.

PLG-1 Expression (VI). To express the PLG-1 protein, the *plg-1* gene was subcloned in an expression vector. The primers "PLG-Exp-S" and "PLG-Exp-E" were designed to include the restriction sites *Nde*I and *Xho*I, respectively. The expression vector pET22b and the PCR product were digested with these restriction enzymes and ligated. This subcloned product would produce a protein containing the polypeptide PLG-1 with 6 histidine residues fused to its C-terminus without an intervening stop codon. The fused gene was under the control of the *lacUV5* promoter. The expression clone was introduced into *E. coli* ER2566. Addition of IPTG to the bacterial culture induced expression of PLG-1. Expression of PLG-1 increased with time and after 3 hr induction the fused protein was more than 50% of total bacterial protein (Fig. 6).

Purification of fused protein was conducted in one step by metal affinity chromatography taking advantage of the high affinity of the 6-His tag to metal ions. The protein was purified under both native and denaturing conditions (Fig. 7); no difference was observed for protein purified by either method. SDS-PAGE analysis indicated that the product was homogeneous. The sequence of the fused protein matched that deduced from the *plg-1* gene.

E. Scientific Implications:

This study demonstrates that propionicin PLG-1 is immunogenic and that antibodies against this protein can be raised successfully in rabbits. The availability of antibodies makes it possible to use immunoassays to follow protein production in a culture, examine large numbers of other cultures for the production of cross-reactive proteins, localize bacteriocin molecules in or on target cells, and follow the introduction of bacteriocin gene(s) into recipient strains by various gene transfer methods. Results here indicated that many strains of propionibacteria produce proteins that cross-react with anti-PLG-1 antibodies. However, biological activity was only detected in a few strains. The nature of the cross-reactive proteins, and the relationship of the larger forms of these proteins to the PLG-1 monomer needs to be explored.

An unexpected use of propionicin PLG-1 antibodies was in successfully screening a *P. jensenii/thoenii* P126 genomic expression library. PLG-1 antibodies identified several possible clones. Complete sequencing of the insert of one clone revealed similarities to other bacteriocin operons. It contained a putative response regulator, transport protein, transmembrane protein and an open reading frame potentially encoding jenseniin G. The molecular weight and isoelectric point of the deduced amino acid sequence agreed with values reported for jenseniin G. Although further confirmation is

required, the *jnG* structural gene and related genes affecting jenseniin G production and transport appear to have been located.

Use of PCR technology allowed cloning of propionicin PLG-1.

F. Potential impact on agriculture, industry, environment, policy, legislation:

In earlier research efforts supported by BARD (US-2080-91), propionicin PLG-1 and jenseniin G were the first bacteriocins to be identified, purified and characterized for the industrially important dairy propionibacteria. In this study, demonstrating that antibodies can be produced for propionicin PLG-1 and that those antibodies can be used to detect, monitor and compare activity throughout growth and purification was an important step towards the use of monitoring their concentrations in food systems. The unexpected but fortunate cross-reactivity of propionicin PLG-1 antibodies with jenseniin G led to their application in selectively recovering jenseniin G by immunoprecipitation. Further refinement of this separation technique could lead to powerful affinity methods for rapid, specific separation of the two bacteriocins and thus facilitate their availability for agricultural, industrial or pharmaceutical uses.

Preliminary identification of genes encoding the two dairy propionibacteria bacteriocins has further defined their characteristics. Once their identity has been confirmed, further analysis will provide means for understanding how they work, for increasing their production and for manipulating the peptides to increase their target species.

The production of these bacteriocins by food-grade organisms, their stability to heat, to extremes of pH, and to storage conditions, and their spectrum of activity make them good candidates for use as food preservatives. Sufficient data (spectrum, storage stability, production conditions, crude preparations, gross mode of action, purification methods, etc.) have been developed for both bacteriocins. Dr. Glatz previously described the use of milk culture fermentates containing propionicin PLG-1 as a preservative in a dairy product. However, before either purified bacteriocin can be applied to foods, clinical and/or toxicological studies would be required.

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32. Weinbrenner, D. R., S. F. Barefoot and D. A. Grinstead. 1997. Inhibition of yogurt starter cultures by jenseniin G, a *Propionibacterium* bacteriocin. J. Dairy Sci. 80:1246-1253.

H. List of Publications or Patents Resulting from the Project:

Refereed Publications:

Ekinci, F.Y. and S.F. Barefoot. 1999. Production of bacteriocin jenseniin G by *Propionibacterium* is pH sensitive. Lett. Appl. Microbiol. 29 (3): 176-180.

Paik, H.D. and B. A. Glatz. 1997. Enhanced bacteriocin production by *Propioni-bacterium thoenii* in fed-batch fermentation. J. Food Prot. 60 (12):1529-1533.

Ratnam, P., S.F. Barefoot, L.D. Prince, L.H. McCaskill and A.B. Bodine. 1999. Identification and partial purification of a bacteriocin produced by *Propionibacterium jensenii* B1264. Lait 79: 12-17.

Weinbrenner, D.R., S.F. Barefoot and D.A. Grinstead. 1997. Inhibition of yogurt starter cultures by jenseniin G, a *Propionibacterium* bacteriocin. J. Dairy Sci. 89(7):1246-1253.

Abstracts:

Baker, S.H., F.Y. Ekinci and S.F. Barefoot. 1999. Bacteriocins of propionibacteria. Symp. Abst. Joint meeting, American Society for Microbiology South Carolina and Southeastern Branches, Jekyll Island, GA. Oct 30-Nov 1.

Barefoot, S.F., K. I. Garver, P. R. Ratnam, D.A. Grinstead and L.D. Prince. 1995. Jenseniins G and P, heat-stable bacteriocins produced by *Propionibacterium jensenii.* Abst. C-13. 1st International Symposium on Propionibacteria, Rennes, France, May 17-19.

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Ekinci, F.Y. and S.F. Barefoot. 2000. Fed-batch enhancement of jenseniin G, a bacteriocin produced by *Propionibacterium jensenii (thoenii)* P126. Abst. Annual Meeting, Institute of Food Technologists, Dallas TX, June 10-14.

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Gollop, N. and P. Lindner. 1998. The bacteriocin PLG-1, an antibacterial peptide with a unique mode of action. Abst. 2nd International Symposium on Propionibacteria, University College, Cork, Ireland. June 25-27.

Leversee, J. A. and B. A. Glatz. 1999. Development of an enzyme-linked immunosorbent assay for the bacteriocin propionicin PLG-1. Abst. 99th Annual Meeting, American Society for Microbiology, May 1999.

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Patents:

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Papers Submitted, in Review, in Preparation:

Baker, S.H., F.Y. Ekinci and S.F. Barefoot. Gene encoding the propionibacteria bacteriocin, jenseniin G. Appl. Environ. Microbiol. In preparation.

Gollop, N. and P. Lindner. The bacteriocin PLG-1, an antibacterial peptide with a unique mode of action. J. Appl. Bacteriol. Submitted for publication.

Leversee, J. A. and B. A. Glatz. Development of an enzyme-linked immunosorbent assay for the bacteriocin propionicin PLG-1. J. Food Prot. In preparation.

Ratnam, P., and S.F. Barefoot. Anti-acne activity of jenseniin P, a bacteriocin produced by a dairy propionibacteria. J. Clin. Micobiol. In preparation.

III. Appendices

Table 1. Cross-reactive propionibacteria strains as determined by indirect ELISA (ISU)

Propionibacterium Species	Number of strains tested	Number of cross-reactive strains (5 or 12 day incubation)
P. thoenii	8	8
P. jensenii	26	18
P. acidipropionici	21	1
P. freudenreichii		
subsp. *shermanii*	79	7
P. freudenreichii		
subsp. *freudenreichii*	14	2
Not classified	10	3
Total	158	39

Table 2. Presence of cross-reactive proteins in supernatants of propionibacteria strains (ISU)

Species/Strain	Days of incubation	Biological activity	Western Blot 16.2 kDa band	Western Blot 27.5 kDa band
P. thoenii				
P4	5	-	+	-
P15	12	+	+	-
P20	12	+	+	-
P30	12	-	+	+
P38	12	-	+	+
P85	12	-	+	-
P126	12	+	+	-
P127	12	+	+	-
P. jensenii				
P10	5	-	-	+
P17	5	-	+	+
P21	5	-	+	+
P25	12	-	-	+
P26	12	-	-	+
P34	12	-	+	+
P41	12	-	-	+
P44	12	-	-	+
P46	12	-	+	+
P52	12	-	-	+
P53	12	-	-	+
P69	12	-	-	+
P105	5	-	+	+
P107	12	-	-	+
P108	12	-	+	+
P111	12	-	-	+
P124	12	-	-	+
P129	5	-	+	-
P. acidipropionici				
P125	12	-	+	-

P. freudenreichii subsp. *shermanii*

P18	12	-	+	-
P35	12	-	+	-
P47	12	-	+	+
P109	12	-	-	-
P110	12	-	+	+
P159	12	-	-	-
P186	12	-	+	+
P. freudenreichii subsp. *freudenreichii*				
P113	12	-	+	-
Not classified				
P180	12	-	-	+
P183	12	-	+	+
P187	12	-	+	+

Table 3. Inhibition of jenseniin G activity by anti-propionicin PLG-1 antibodies

Sample	**Zone of inhibition (cm)**
Anti-propionicin PLG-1 antibodies only	0
PIS[a] + 100 AU/ml jenseniin G	0.9
1:100 antibodies + 100 AU/ml jenseniin G	0
1:1000 antibodies + 100 AU/ml jenseniin G	0
1:10,000 antibodies + 100 AU/ml jenseniin G	0
PIS + 1000 AU/ml jenseniin G	1.6
1:100 antibodies + 1000 AU/ml jenseniin G	0.9
1:1000 antibodies + 1000 AU/ml jenseniin G	1.5
1:10,000 antibodies + 1000 AU/ml jenseniin G	1.6

[a] PIS = preimmune serum.